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(54) Title: INDIVIDUALIZED ANTI-CANCER ANTIBODIES

(57) Abstract: The present invention relates to a method for producing patient specific anti-cancer antibodies using a novel paradigm of screening. By segregating the anti-cancer antibodies using cancer cell cytotoxicity as an end point, the process makes possible the production of anti-cancer antibodies customized for the individual patient that can be used for therapeutic and diagnostic purposes. The invention further relates to the process by which the antibodies are made and to their methods of use. The antibodies can be made specifically for one tumor derived from a particular patient and are selected on the basis of their cancer cell cytotoxicity and simultaneous lack of toxicity for non-cancerous cells. The antibodies can be used in aid of staging and diagnosis of a cancer, and can be used to treat tumor metastases. The anti-cancer antibodies can be conjugated to red blood cells obtained from that patient and re-infused for treatment of metastases based upon the recognition that metastatic cancers are usually well vascularized and the delivery of anti-cancer antibodies by red blood cells can have the effect of concentrating the antibodies at the site of the tumor.

INDIVIDUALIZED ANTI-CANCER ANTIBODIES**Field of the Invention:**

This invention relates to the production of anti-cancer antibodies customized for the individual patient that can be used for therapeutic and diagnostic purposes. The invention further relates to the process by which the antibodies are made and to their methods of use.

Background of the Invention:

Each individual who presents with cancer is unique and has a cancer that is as different from other cancers as that person's identity. Despite this, current therapy treats all patients with the same type of cancer, at the same stage, in the same way. At least 30% of these patients will fail the first line therapy, thus leading to further rounds of treatment and the increased probability of treatment failure, metastases, and ultimately, death. A superior approach to treatment would be the customization of therapy for the particular individual. The only current therapy which lends itself to customization is surgery. Chemotherapy and radiation treatment can not be tailored to the patient, and surgery by itself, in most cases is inadequate for producing cures.

With the advent of monoclonal antibodies, the possibility of developing methods for customized therapy became more realistic since each antibody can be directed to a single epitope. Furthermore, it is possible to produce a combination of antibodies that are directed to the constellation of epitopes that uniquely define a particular individual's tumor.

Having recognized that a significant difference between cancerous and normal cells is that cancerous cells contain antigens that are specific to transformed cells, the scientific community has long held that monoclonal

1 antibodies can be designed to specifically target
2 transformed cells by binding specifically to these cancer
3 antigens; thus giving rise to the belief that monoclonal
4 antibodies can serve as "Magic Bullets" to eliminate
5 cancer cells.

6 At the present time, however, the cancer patient
7 usually has few options of treatment. The regimented
8 approach to cancer therapy has produced improvements in
9 global survival and morbidity rates. However, to the
10 particular individual, these improved statistics do not
11 necessarily correlate with an improvement in their
12 personal situation.

13 Thus, if a methodology was put forth which enabled
14 the practitioner to treat each tumor independently of
15 other patients in the same cohort, this would permit the
16 unique approach of tailoring therapy to just that one
17 person. Such a course of therapy would, ideally, increase
18 the rate of cures, and produce better outcomes, thereby
19 satisfying a long-felt need.

20 Historically, the use of polyclonal antibodies has
21 been used with limited success in the treatment of human
22 cancers. Lymphomas and leukemias have been treated with
23 human plasma, but there were few prolonged remission or
24 responses. Furthermore, there was a lack of
25 reproducibility and there was no additional benefit
26 compared to chemotherapy. Solid tumors such as breast
27 cancers, melanomas and renal cell carcinomas have also
28 been treated with human blood, chimpanzee serum, human
29 plasma and horse serum with correspondingly unpredictable
30 and ineffective results.

31 There have been many clinical trials of monoclonal
32 antibodies for solid tumors. In the 1980s there were at
33 least four clinical trials for human breast cancer which
34 produced only one responder from at least 47 patients
35 using antibodies against specific antigens or based on
36 tissue selectivity. It was not until 1998 that there was

1 a successful clinical trial using a humanized anti-her 2
2 antibody in combination with Cisplatin. In this trial 37
3 patients were accessed for responses of which about a
4 quarter had a partial response rate and another half had
5 minor or stable disease progression.

6 The clinical trials investigating colorectal cancer
7 involve antibodies against both glycoprotein and
8 glycolipid targets. Antibodies such as 17-1A, which has
9 some specificity for adenocarcinomas, had undergone Phase
10 2 clinical trials in over 60 patients with only one
11 patient having a partial response. In other trials, use
12 of 17-1A produced only one complete response and two minor
13 responses among 52 patients in protocols using additional
14 cyclophosphamide. Other trials involving 17-1A yielded
15 results that were similar. The use of a humanized murine
16 monoclonal antibody initially approved for imaging also
17 did not produce tumor regression. To date there has not
18 been an antibody that has been effective for colorectal
19 cancer. Likewise there have been equally poor results for
20 lung cancer, brain cancers, ovarian cancers, pancreatic
21 cancer, prostate cancer, and stomach cancer. There has
22 been some limited success in the use of anti-GD3
23 monoclonal antibody for melanoma. Thus, it can be seen
24 that despite successful small animal studies that are a
25 prerequisite for human clinical trials, the antibodies
26 that have been tested have been for the most part
27 ineffective.

28 **Prior Patents:**

29 U.S. Patent No. 5,750,102 discloses a process wherein
30 cells from a patient's tumor are transfected with MHC
31 genes which may be cloned from cells or tissue from the
32 patient. These transfected cells are then used to
33 vaccinate the patient.

34 U.S. Patent No. 4,861,581 discloses a process
35 comprising the steps of obtaining monoclonal antibodies
36 that are specific to an internal cellular component of

1 neoplastic and normal cells of the mammal but not to
2 external components, labeling the monoclonal antibody,
3 contacting the labeled antibody with tissue of a mammal
4 that has received therapy to kill neoplastic cells, and
5 determining the effectiveness of therapy by measuring the
6 binding of the labeled antibody to the internal cellular
7 component of the degenerating neoplastic cells. In
8 preparing antibodies directed to human intracellular
9 antigens, the patentee recognizes that malignant cells
10 represent a convenient source of such antigens.

11 U.S. Patent No. 5,171,665 provides a novel antibody
12 and method for its production. Specifically, the patent
13 teaches formation of a monoclonal antibody which has the
14 property of binding strongly to a protein antigen
15 associated with human tumors, e.g. those of the colon and
16 lung, while binding to normal cells to a much lesser
17 degree.

18 U.S. Patent No. 5,484,596 provides a method of cancer
19 therapy comprising surgically removing tumor tissue from a
20 human cancer patient, treating the tumor tissue to obtain
21 tumor cells, irradiating the tumor cells to be viable but
22 non-tumorigenic, and using these cells to prepare a
23 vaccine for the patient capable of inhibiting recurrence
24 of the primary tumor while simultaneously inhibiting
25 metastases. The patent teaches the development of
26 monoclonal antibodies which are reactive with surface
27 antigens of tumor cells. As set forth at col. 4, lines 45
28 et seq., the patentees utilize autochthonous tumor cells
29 in the development of monoclonal antibodies expressing
30 active specific immunotherapy in human neoplasia.

31 U.S. Patent No. 5,693,763 teaches a glycoprotein
32 antigen characteristic of human carcinomas and not
33 dependent upon the epithelial tissue of origin.

34 U.S. Patent No. 5,783,186 is drawn to Anti-Her2
35 antibodies which induce apoptosis in Her2 expressing
36 cells, hybridoma cell lines producing the antibodies,

1 methods of treating cancer using the antibodies and
2 pharmaceutical compositions including said antibodies.

3 U.S. Patent No. 5,849,876 describes new hybridoma
4 cell lines for the production of monoclonal antibodies to
5 mucin antigens purified from tumor and non-tumor tissue
6 sources.

7 U.S. Patent No. 5,869,268 is drawn to a method for
8 producing a human lymphocyte producing an antibody
9 specific to a desired antigen, a method for producing a
10 monoclonal antibody, as well as monoclonal antibodies
11 produced by the method. The patent is particularly drawn
12 to the production of an anti-HD human monoclonal antibody
13 useful for the diagnosis and treatment of cancers.

14 U.S. Patent No. 5,869,045 relates to antibodies,
15 antibody fragments, antibody conjugates and single chain
16 immunotoxins reactive with human carcinoma cells. The
17 mechanism by which these antibodies function is two-fold,
18 in that the molecules are reactive with cell membrane
19 antigens present on the surface of human carcinomas, and
20 further in that the antibodies have the ability to
21 internalize within the carcinoma cells, subsequent to
22 binding, making them especially useful for forming
23 antibody-drug and antibody-toxin conjugates. In their
24 unmodified form the antibodies also manifest cytotoxic
25 properties at specific concentrations.

26 U.S. Patent No. 5,780,033 discloses the use of
27 autoantibodies for tumor therapy and prophylaxis. However,
28 this antibody is an antinuclear autoantibody from an aged
29 mammal. In this case, the autoantibody is said to be one
30 type of natural antibody found in the immune system.
31 Because the autoantibody comes from "an aged mammal",
32 there is no requirement that the autoantibody actually
33 comes from the patient being treated. In addition the
34 patent discloses natural and monoclonal antinuclear
35 autoantibody from an aged mammal, and a hybridoma cell
36 line producing a monoclonal antinuclear autoantibody.

1 **Summary of the Invention:**

2 This application teaches a method for producing
3 patient specific anti-cancer antibodies using a novel
4 paradigm of screening. These antibodies can be made
5 specifically for one tumor and thus make possible the
6 customization of cancer therapy. Within the context of
7 this application, anti-cancer antibodies having either
8 cell-killing (cytotoxic) or cell-growth inhibiting
9 (cytostatic) properties will hereafter be referred to as
10 cytotoxic. These antibodies can be used in aid of staging
11 and diagnosis of a cancer, and can be used to treat tumor
12 metastases.

13 The prospect of individualized anti-cancer treatment
14 will bring about a change in the way a patient is managed.
15 A likely clinical scenario is that a tumor sample is
16 obtained at the time of presentation, and banked. From
17 this sample, the tumor can be typed from a panel of pre-
18 existing anti-cancer antibodies. The patient will be
19 conventionally staged but the available antibodies can be
20 of use in further staging the patient. The patient can be
21 treated immediately with the existing antibodies, and a
22 panel of antibodies specific to the tumor can be produced
23 either using the methods outlined herein or through the
24 use of phage display libraries in conjunction with the
25 screening methods herein disclosed. All the antibodies
26 generated will be added to the library of anti-cancer
27 antibodies since there is a possibility that other tumors
28 can bear some of the same epitopes as the one that is
29 being treated.

30 In addition to anti-cancer antibodies, the patient
31 can elect to receive the currently recommended therapies
32 as part of a multi-modal regimen of treatment. The fact
33 that the antibodies isolated via the present methodology
34 are relatively non-toxic to non-cancerous cells allow
35 combinations of antibodies at high doses to be used,
36 either alone, or in conjunction with conventional therapy.

1 The high therapeutic index will also permit re-treatment
2 on a short time scale that should decrease the likelihood
3 of emergence of treatment resistant cells.

4 If the patient is refractory to the initial course of
5 therapy or metastases develop, the process of generating
6 specific antibodies to the tumor can be repeated for re-
7 treatment. Furthermore, the anti-cancer antibodies can be
8 conjugated to red blood cells obtained from that patient
9 and re-infused for treatment of metastases. There have
10 been few effective treatments for metastatic cancer and
11 metastases usually portend a poor outcome resulting in
12 death. However, metastatic cancers are usually well
13 vascularized and the delivery of anti-cancer antibodies by
14 red blood cells can have the effect of concentrating the
15 antibodies at the site of the tumor. Even prior to
16 metastases, most cancer cells are dependent on the host's
17 blood supply for their survival and anti-cancer antibody
18 conjugated red blood cells can be effective against *in*
19 *situ* tumors, too. Alternatively, the antibodies may be
20 conjugated to other hematogenous cells, e.g. lymphocytes,
21 macrophages, monocytes, natural killer cells, etc.

22 There are five classes of antibodies and each is
23 associated with a function that is conferred by its heavy
24 chain. It is generally thought that cancer cell killing
25 by naked antibodies are mediated either through antibody
26 dependent cellular cytotoxicity or complement dependent
27 cytotoxicity. For example murine IgM and IgG2a antibodies
28 can activate human complement by binding the C-1 component
29 of the complement system thereby activating the classical
30 pathway of complement activation which can lead to tumor
31 lysis. For human antibodies the most effective complement
32 activating antibodies are generally IgM and IgG1. Murine
33 antibodies of the IgG2a and IgG3 isotype are effective at
34 recruiting cytotoxic cells that have Fc receptors which
35 will lead to cell killing by monocytes, macrophages,

1 granulocytes and certain lymphocytes. Human antibodies of
2 both the IgG1 and IgG3 isotype mediate ADCC.

3 Another possible mechanism of antibody mediated
4 cancer killing may be through the use of antibodies that
5 function to catalyze the hydrolysis of various chemical
6 bonds in the cell membrane and its associated
7 glycoproteins or glycolipids, so-called catalytic
8 antibodies.

9 There are two additional mechanisms of antibody
10 mediated cancer cell killing which are more widely
11 accepted. The first is the use of antibodies as a vaccine
12 to induce the body to produce an immune response against
13 the putative cancer antigen that resides on the tumor
14 cell. The second is the use of antibodies to target
15 growth receptors and interfere with their function or to
16 down regulate that receptor so that effectively its
17 function is lost.

18 Accordingly, it is an objective of the invention to
19 teach a method for producing anti-cancer antibodies from
20 cells derived from a particular individual which are
21 cytotoxic with respect to cancer cells while
22 simultaneously being relatively non-toxic to non-cancerous
23 cells.

24 It is an additional objective of the invention to
25 produce novel anti-cancer antibodies.

26 It is a further objective of the instant invention to
27 produce anti-cancer antibodies whose cytotoxicity is
28 mediated through antibody dependent cellular toxicity.

29 It is yet an additional objective of the instant
30 invention to produce anti-cancer antibodies whose
31 cytotoxicity is mediated through complement dependent
32 cellular toxicity.

33 It is still a further objective of the instant
34 invention to produce anti-cancer antibodies whose

1 cytotoxicity is a function of their ability to catalyze
2 hydrolysis of cellular chemical bonds.

3 Still an additional objective of the instant
4 invention is to produce anti-cancer antibodies useful as a
5 vaccine to produce an immune response against putative
6 cancer antigen residing on tumor cells.

7 A further objective of the instant invention is the
8 use of antibodies to target cell membrane proteins, such
9 as growth receptors, cell membrane pumps and cell
10 anchoring proteins, thereby interfering with or down
11 regulating their function.

12 Yet an additional objective of the instant invention
13 is the production of anti-cancer antibodies whose cell-
14 killing utility is concomitant with their ability to
15 effect a conformational change in cellular proteins such
16 that a signal will be transduced to initiate cell-killing.

17 A still further objective of the instant invention is
18 to produce anti-cancer antibodies which are useful for
19 diagnosis, prognosis, and monitoring of cancer, e.g.
20 production of a panel of therapeutic anti-cancer
21 antibodies to test patient samples to determine if they
22 contain any suitable antibodies for therapeutic use.

23 Yet another objective of the instant invention is to
24 produce novel antigens, associated with cancer processes,
25 which can be discovered by using anti-cancer antibodies
26 derived by the process of the instant invention. These
27 antigens are not limited to proteins, as is generally the
28 case with genomic data; they may also be derived from
29 carbohydrates or lipids or combinations thereof.

30 Other objects and advantages of this invention will
31 become apparent from the following description wherein are
32 set forth, by way of illustration and example, certain
33 embodiments of this invention.

34

35

1 **Detailed Description of the Invention:**

2 It is to be understood that while a certain form of
3 the invention is illustrated, it is not to be limited to
4 the specific form or arrangement herein described and
5 shown. It will be apparent to those skilled in the art
6 that various changes may be made without departing from
7 the scope of the invention and the invention is not to be
8 considered limited to what is shown and described in the
9 specification.

10 One of the potential benefits of monoclonal
11 antibodies with respect to the treatment of cancer is
12 their ability to specifically recognize single antigens.
13 It was thought that in some instances cancer cells possess
14 antigens that were specific to that kind of transformed
15 cell. It is now more frequently believed that cancer
16 cells have few unique antigens, rather, they tend to over-
17 express a normal antigen or express fetal antigens.
18 Nevertheless, the use of monoclonal antibodies provided a
19 method of delivering reproducible doses of antibodies to
20 the patient with the expectation of better response rates
21 than with polyclonal antibodies.

22 Traditionally, monoclonal antibodies have been made
23 according to fundamental principles laid down by Kohler
24 and Milstein. Mice are immunized with antigens, with or
25 without, adjuvants. The splenocytes are harvested from
26 the spleen for fusion with immortalized hybridoma
27 partners. These are seeded into microtitre plates where
28 they can secrete antibodies into the supernatant that is
29 used for cell culture. To select from the hybridomas that
30 have been plated for the ones that produce antibodies of
31 interest the hybridoma supernatants are usually tested for
32 antibody binding to antigens in an ELISA (enzyme linked
33 immunosorbent assay) assay. The idea is that the wells
34 that contain the hybridoma of interest will contain
35 antibodies that will bind most avidly to the test antigen,
36 usually the immunizing antigen. These wells are then

1 subcloned in limiting dilution fashion to produce
2 monoclonal hybridomas. The selection for the clones of
3 interest is repeated using an ELISA assay to test for
4 antibody binding. Therefore, the principle that has been
5 propagated is that in the production of monoclonal
6 antibodies the hybridomas that produce the most avidly
7 binding antibodies are the ones that are selected from
8 among all the hybridomas that were initially produced.
9 That is to say, the preferred antibody is the one with
10 highest affinity for the antigen of interest.

11 There have been many modifications of this procedure
12 such as using whole cells for immunization. In this
13 method, instead of using purified antigens, entire cells
14 are used for immunization. Another modification is the
15 use of cellular ELISA for screening. In this method
16 instead of using purified antigens as the target in the
17 ELISA, fixed cells are used. In addition to ELISA tests,
18 complement mediated cytotoxicity assays have also been
19 used in the screening process. However, antibody-binding
20 assays were used in conjunction with cytotoxicity tests.
21 Thus, despite many modifications, the process of producing
22 monoclonal antibodies relies on antibody binding to the
23 test antigen as an endpoint.

24 Most antibodies directed against cancer cells have
25 been produced using the traditional methods outlined
26 above. These antibodies have been used both
27 therapeutically and diagnostically. In general, for both
28 these applications, the antibody has been used as the
29 targeting agent that delivers a payload to the site of the
30 cancer. These antibody conjugates can either be
31 radioactive, toxic, or serve as an intermediary for
32 further delivery of a drug to the body, such as an enzyme
33 or biotin. Furthermore, it was widely held, until
34 recently, that naked antibodies had little effect *in vivo*.
35 Both HERCEPTIN and RITUXIMAB are humanized murine
36 monoclonal antibodies that have recently been approved for

1 human use by the FDA. However, both these antibodies were
2 initially made by assaying for antibody binding and their
3 direct cytotoxicity was not the primary goal during the
4 production of hybridomas. Any tendency for these
5 antibodies to produce tumor cell killing is thus through
6 chance, not by design.

7 Although the production of monoclonal antibodies have
8 been carried out using whole cell immunization for various
9 applications the screening of these hybridomas have relied
10 on either putative or identified target antigens or on the
11 selectivity of these hybridomas for specific tissues. It
12 is axiomatic that the best antibodies are the ones with
13 the highest binding constants. This concept originated
14 from the basic biochemical principle that enzymes with the
15 highest binding constants were the ones that were the most
16 effective for catalyzing a reaction. This concept is
17 applicable to receptor ligand binding where the drug
18 molecule binding to the receptor with the greatest
19 affinity usually has the highest probability for
20 initiating or inhibiting a signal. However, this may not
21 always be the case since it is possible that in certain
22 situations there may be cases where the initiation or
23 inhibition of a signal may be mediated through non-
24 receptor binding. The information conveyed by a
25 conformational change induced by ligand binding can have
26 many consequences such as a signal transduction,
27 endocytosis, among the others. The ability to produce a
28 conformational change in a receptor molecule may not
29 necessarily be due to the filling of a ligand receptor
30 pocket but may occur through the binding of another extra
31 cellular domain or due to receptor clustering induced by a
32 multivalent ligand.

33 The production of antibodies to produce cell killing
34 need not be predicated upon screening of the hybridomas
35 for the best binding antibodies. Rather, although not
36 advocated by those who produce monoclonal antibodies, the

1 screening of the hybridoma supernatants for cell killing
2 or alternatively for cessation of growth of the cancerous
3 cells may be selected as a desirable endpoint for the
4 production of cytotoxic or cytostatic antibodies. It is
5 well understood that the *in-vivo* antibodies mediate their
6 function through the Fc portions and that the utility of
7 the therapeutic antibody is determined by the
8 functionality of the constant region or attached moieties.
9 In this case the FAb portion of the antibody, the antigen-
10 combining portion, will confer to the antibody its
11 specificity and the Fc portion its functionality. The
12 antigen combining site of the antibody can be considered
13 to be the product of a natural combinatorial library. The
14 result of the rearrangement of the variable region of the
15 antibody can be considered a molecular combinatorial
16 library where the output is a peptide. Therefore, the
17 sampling of this combinatorial library can be based on any
18 parameter. Like sampling a natural compound library for
19 antibiotics, it is possible to sample an antibody library
20 for cytotoxic or cytostatic compounds.

21 The various endpoints in a screen must be
22 differentiated from each other. For example, the
23 difference between antibody binding to the cell is
24 distinct from cell killing. Cell killing (cytotoxicity) is
25 distinct from the mechanisms of cell death such as oncosis
26 or apoptosis. There can be many processes by which cell
27 death is achieved and some of these can lead either to
28 oncosis or apoptosis. There is speculation that there are
29 other cell death mechanisms other than oncosis or
30 apoptosis but regardless of how the cell arrives at death
31 there are some commonalities of cell death. One of these
32 is the absence of metabolism and another is the
33 denaturation of enzymes. In either case vital stains will
34 fail to stain these cells. These endpoints of cell death
35 have been long understood and predate the current
36 understanding of the mechanisms of cell death.

1 Furthermore, there is the distinction between cytotoxic
2 effects where cells are killed and cytostatic effects
3 where the proliferation of cells are inhibited.

4 In a preferred embodiment of the present invention,
5 the assay is conducted by focusing on cytotoxic activity
6 toward cancerous cells as an end point. In a preferred
7 embodiment, a live /dead assay kit , for example the
8 LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L-3224) by
9 Molecular Probes, is utilized. The Molecular Probes kit
10 provides a two-color fluorescence cell viability assay
11 that is based on the simultaneous determination of live
12 and dead cells with two probes that measure two recognized
13 parameters of cell viability - intracellular esterase
14 activity and plasma membrane integrity. The assay
15 principles are general and applicable to most eukaryotic
16 cell types, including adherent cells and certain tissues,
17 but not to bacteria or yeast. This fluorescence-based
18 method of assessing cell viability is preferred in place
19 of such assays as trypan blue exclusion, Cr release and
20 similar methods for determining cell viability and
21 cytotoxicity.

22 In carrying out the assay, live cells are
23 distinguished by the presence of ubiquitous intracellular
24 esterase activity, determined by the enzymatic conversion
25 of the virtually nonfluorescent cell-permeant CALCEIN AM
26 to the intensely fluorescent Calcein. The polyanionic dye
27 Calcein is well retained within live cells, producing an
28 intense uniform green fluorescence in live cells (ex/em
29 ~495 nm/~515 nm). EthD-1 enters cells with damaged
30 membranes and undergoes a 40-fold enhancement of
31 fluorescence upon binding to nucleic acids, thereby
32 producing a bright red fluorescence in dead cells (ex/em
33 ~495 nm/~635 nm). EthD-1 is excluded by the intact plasma
34 membrane of live cells. The determination of cell
35 viability depends on these physical and biochemical
36 properties of cells. Cytotoxic events that do not affect

1 these cell properties may not be accurately assessed using
2 this method. Background fluorescence levels are inherently
3 low with this assay technique because the dyes are
4 virtually nonfluorescent before interacting with cells.

5 In addition to the various endpoints for screening,
6 there are two other major characteristics of the screening
7 process. The library of antibody gene products is not a
8 random library but is the product of a biasing procedure.
9 In the examples below, the biasing is produced by
10 immunizing mice with fixed cells. This increases the
11 proportion of antibodies that have the potential to bind
12 the target antigen. Although immunization is thought of as
13 a way to produce higher affinity antibodies (affinity
14 maturation) in this case it is not. Rather, it can be
15 considered as a way to shift the set of antigen combining
16 sites towards the targets. This is also distinct from the
17 concept of isotype switching where the functionality, as
18 dictated by the constant portion of the heavy chain, is
19 altered from the initial IgM isotype to another isotype
20 such as IgG.

21 The third key feature that is crucial in the
22 screening process is the use of multitarget screening. To
23 a certain extent specificity is related to affinity. An
24 example of this is the situation where an antigen has very
25 limited tissue distribution and the affinity of the
26 antibody is a key determinant of the specificity of the
27 antibody-the higher the affinity the more tissue specific
28 the antibody and likewise an antibody with low affinity
29 may bind to tissues other than the one of interest.
30 Therefore, to address the specificity issue the antibodies
31 are screened simultaneously against a variety of cells. In
32 the examples below the hybridoma supernatants
33 (representing the earliest stages of monoclonal antibody
34 development), are tested against a number of cell lines to
35 establish specificity as well as activity.

1 The antibodies are designed for therapeutic treatment
2 of cancer in patients. Ideally the antibodies can be naked
3 antibodies. They can also be conjugated to toxins. They
4 can be used to target other molecules to the cancer. e.g.
5 biotin conjugated enzymes. Radioactive compounds can also
6 be used for conjugation.

7 The antibodies can be fragmented and rearranged
8 molecularly. For example Fv fragments can be made; sFv-
9 single chain Fv fragments; diabodies etc.

10 It is envisioned that these antibodies can be used
11 for diagnosis, prognosis, and monitoring of cancer. For
12 example the patients can have blood samples drawn for shed
13 tumor antigens which can be detected by these antibodies
14 in different formats such as ELISA assays, rapid test
15 panel formats etc. The antibodies can be used to stain
16 tumor biopsies for the purposes of diagnosis. In addition
17 a panel of therapeutic antibodies can be used to test
18 patient samples to determine if there are any suitable
19 antibodies for therapeutic use.

20 **Example one**

21 In order to produce monoclonal antibodies specific
22 for a tumor sample the method of selection of the
23 appropriate hybridoma wells is complicated by the
24 probability of selecting wells which will produce false
25 positive signals. That is to say that there is the
26 likelihood of producing antibodies that can react against
27 normal cells as well as cancer cells. To obviate this
28 possibility one strategy is to mask the anti-normal
29 antigen antibodies from the selection process. This can
30 be accomplished by removing the anti-normal antibodies at
31 the first stage of screening thereby revealing the
32 presence of the desired antibodies. Subsequent limiting
33 dilution cloning can delineate the clones that will not
34 produce killing of control cells but will produce target
35 cancer cell killing.

1 Biopsy specimens of breast, melanoma, and lung tumors
2 were obtained and stored at -70°C until used. Single cell
3 suspensions were prepared and fixed with -30°C, 70%
4 ethanol, washed with PBS and reconstituted to an
5 appropriate volume for injection. Balb/c mice were
6 immunized with 2.5×10^5 - 1×10^6 cells and boosted every third
7 week until a final pre-fusion boost was performed three
8 days prior to the splenectomy. The hybridomas were
9 prepared by fusing the isolated splenocytes with Sp2/0 and
10 NS1 myeloma partners. The supernatants from the fusions
11 were tested for subcloning of the hybridomas.

12 Cells (including A2058 melanoma cells, CCD-12CoN
13 fibroblasts, MCF-12A breast cells among others) were
14 obtained from ATCC and cultured according to enclosed
15 instructions. The HEY cell line was a gift from Dr. Inka
16 Brockhausen. The non-cancer cells, e.g. CCD-12CoN
17 fibroblasts and MCF-12A breast cells, were plated into 96-
18 well microtitre plates (NUNC) 1 to 2 weeks prior to
19 screening. The cancer cells, e.g. HEY, A2058, BT 483, and
20 HS294t, were plated two or three days prior to screening.

21 The plated normal cells were fixed prior to use. The
22 plates were washed with 100 microliters of PBS for 10
23 minutes at room temperature and then aspirated dry. 75
24 microliters of 0.01 percent glutaraldehyde diluted in PBS
25 were added to each well for five minutes and then
26 aspirated. The plates were washed with 100 microliters of
27 PBS three times at room temperature. The wells were
28 emptied and 100 microliters of one percent human serum
29 albumin in PBS was added to each well for one hour at room
30 temperature. The plates were then stored at four degrees
31 Celsius.

32 Prior to the transfer of the supernatant from the
33 hybridoma plates the fixed normal cells were washed three
34 times with 100 microliters of PBS at room temperature.
35 After aspiration to the microliters of the primary
36 hybridoma culture supernatants were transferred to the

1 fixed cell plates and incubated for two hours at 37
2 degrees Celsius in a 8 percent CO₂ incubator. The
3 hybridoma supernatants derived from melanoma was incubated
4 with CCD-12 CoN cells and those derived from breast cancer
5 were incubated with MCF-12a cells. After incubation
6 the absorbed supernatant was divided into two 75
7 microliter portions and transferred to target cancer cell
8 plates. Prior to the transfer the cancer cell plates were
9 washed three times with 100 microliters of PBS. The
10 supernatant from the CCD-12 CoN cells were transferred to
11 the A2058 and the HS294t cells, whereas the supernatant
12 from MCF-12A cells were transferred to the HEY and BT 483
13 cells. The cancer cells were incubated with the hybridoma
14 supernatants for 18 hours at 37 degrees Celsius in an 8
15 percent CO₂ incubator.

16 The Live/Dead cytotoxicity assay was obtained from
17 Molecular Probes (Eu,OR). The assays were performed
18 according to the manufacturer's instructions with the
19 changes outlined below. The plates with the cells were
20 washed once with 100 microliters of PBS at 37°C. 75 to 100
21 microliters of supernatant from the hybridoma microtitre
22 plates were transferred to the cell plates and incubated
23 in a 8% CO₂ incubator for 18-24 hours. Then, the wells that
24 served as the all dead control were aspirated until empty
25 and 50 microliters of 70% ethanol was added. The plate was
26 then emptied by inverting and blotted dry. Room
27 temperature PBS was dispensed into each well from a
28 multichannel squeeze bottle, tapped three times, emptied
29 by inversion and then blotted dry. 50 microliters of the
30 fluorescent Live/Dead dye diluted in PBS was added to each
31 well and incubated at 37°C in a 5% CO₂ incubator for one
32 hour. The plates were read in a Perkin-Elmer HTS7000
33 fluorescence plate reader and the data was analyzed in
34 Microsoft Excel.

35 Four rounds of screening were conducted to produce
36 single clone hybridoma cultures. For two rounds of

1 screening the hybridoma supernatants were tested only
2 against the cancer cells. In the last round of screening
3 the supernatant was tested against a number of non-cancer
4 cells as well as the target cells indicated in table 1.
5 The antibodies were isotyped using a commercial isotyping
6 kit.

7 A number of monoclonal antibodies were produced in
8 accordance with the method of the present invention.
9 These antibodies, whose characteristics are summarized in
10 Table 1, are identified as 3BD-3, 3BD-6, 3BD-8, 3BD-9,
11 3BD-15, 3BD-25, 3BD-26 and 3BD-27. These antibodies are
12 considered monoclonal after four rounds of limiting
13 dilution cloning. The anti-melanoma antibodies did not
14 produce significant cancer cell killing. The panel of
15 anti-breast cancer antibodies killed 32-87% of the target
16 cells and <1-3% of the control cells. The predominant
17 isotype was IgG1 even though it was expected that the
18 majority of anti-tumor antibodies would be directed
19 against carbohydrate antigens, and thus, be of the IgM
20 type. There is a high therapeutic index since most
21 antibodies spare the control cells from cell death.

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Table 1. Anti-Breast Cancer Antibodies

Clones	% Cell Death			
	Target for Anti-Breast Cancer Antibodies (HEY & A2058)	Normal Fibroblast Cells (CCD-12CoN)	Fibrocystic Breast Cells (MCF-12A)	Isotype
3BD-3	74.9%	3.7%	<1%	$\gamma 1, \lambda$
3BD-6	68.5%	5.6%	<1%	$\gamma 1, \lambda$
3BD-8	81.9%	4.5%	2.6%	$\gamma 1, \kappa$
3BD-9	77.2%	7.9%	<1%	$\gamma 1, \lambda$
3BD-15	87.1%	<1 %	<1%	$\gamma 1, \lambda$
3BD-26	54.8%	3.3%	<1%	μ, κ
3BD-25	32.4%	3.6%	<1 %	$\gamma 1, \kappa$
3BD-27	60.1%	8.3%	1.3%	$\gamma 1, \kappa$

Example 2

In this example customized anti-cancer antibodies are produced by first obtaining samples of the patient's tumor. Usually this is from a biopsy specimen from a solid tumor or a blood sample from hematogenous tumors. The samples are prepared into single cell suspensions and fixed for injection into mice. After the completion of the immunization schedule the hybridomas are produced from the splenocytes. The hybridomas are screened against a variety of cancer cell lines and normal cells in standard cytotoxicity assays. Those hybridomas that are reactive against cancer cell lines but are not reactive against normal non-transformed cells are selected for further propagation. Clones that were considered positive were ones that selectively killed the cancer cells but did not kill the non-transformed cells. The antibodies are

1 characterized for a large number of biochemical parameters
2 and then humanized for therapeutic use.

3 The melanoma tumor cells isolated and cell lines were
4 cultured as described in Example 1. Balb/c mice were
5 immunized according to the following schedule: 200,000
6 cells s.c. and i.p. on day 0, then 200,000 cells were
7 injected i.p. on day 21, then 1,000,000 cells were
8 injected on day 49, then 1,250,000 cells in Freund's
9 Complete Adjuvant were injected i.p. on day 107, and then
10 200,000 cells were injected on day 120 i.p. and then the
11 mice were sacrificed on day 123. The spleens were
12 harvested and the splenocytes were divided into two
13 aliquots for fusion with Sp2/0 (1LN) or NS-1 (2LN) myeloma
14 partners using the methods outlined in example 1.

15 The screening was carried out 11 days after the
16 fusion against A2058 melanoma cells and CCD-12CoN
17 fibroblasts. Each pair of plates were washed with 100
18 microliters of room temperature PBS and then aspirated to
19 near dryness. Then 50 microliters of hybridoma supernatant
20 was added to the same wells on each of the two plates. The
21 spent Sp2/0 supernatant was added to the control wells at
22 the same volume and the plates were incubated for around
23 18 hours at 37 degrees Celsius at a 8%CO₂, 98% relative
24 humidity incubator. Then each pair of plates were removed
25 and in the positive control wells 50 microliters of 70%
26 ethanol was substituted for the media for 4 seconds. The
27 plates were then inverted and washed with room temperature
28 PBS once and dried. Then 50uL of fluorescent live/dead dye
29 diluted in PBS (Molecular Probes Live/Dead Kit) was added
30 for one hour and incubated at 37 degrees Celsius. The
31 plates were then read in a Perkin-Elmer fluorescent plate
32 reader and the data analyzed using Microsoft Excel. The
33 wells that were considered positive were subcloned and the
34 same screening process was repeated 13 days later and then
35 33 days later. The results of the last screening is
36 outlined in Table 2 below. A number of monoclonal

antibodies were produced in accordance with the method of the present invention. These antibodies, whose characteristics are summarized in Table 2, are identified as 1LN-1, 1LN-12, 1LN-14, 2LN-21, 2LN-28, 2LN-29, 2LN-31, 2LN-33, 2LN-34 and 2LN-35.

Table 2, Anti-Melanoma Antibodies

Clones	% Cell Death	
	Target for Anti-Melanoma Antibodies	Normal Fibroblast Cells
	(A2058)	(CCD-1 2CoN)
1LN-1	59.4%	<1 %
1LN-12	55.2%	1.4%
1LN-14	51.4%	<1%
2LN-21	72.0%	15.9%
2LN-28	66.6%	12.4%
2LN-29	78.2%	6.1%
2LN-31	100%	7.8%
2LN-33	94.2%	<1%
2LN-34	56.6%	11.2%
2LN-35	66.5%	6.6%

The table illustrates that clones from both the Sp2/0 and NS-1 fusions were able to produce antibodies that had a greater than 50% killing rate for cancerous cells and at the same time some of the clones were able to produce less than one percent killing of normal control fibroblasts.

The anti-cancer antibodies of the invention are useful for treating a patient with a cancerous disease when administered in admixture with a pharmaceutically acceptable adjuvant, for example normal saline, a lipid emulsion, albumen, phosphate buffered saline or the like and are administered in an amount effective to mediate treatment of said cancerous disease, for example with a range of about 1 microgram per mil to about 1 gram per mil.

1 The method for treating a patient suffering from a
2 cancerous disease may further include the use of
3 conjugated anti-cancer antibodies and would this include
4 conjugating patient specific anti-cancer antibodies with a
5 member selected from the group consisting of toxins,
6 enzymes, radioactive compounds, and hematogenous cells;
7 and
8 administering these conjugated antibodies to the patient;
9 wherein said anti-cancer antibodies are administered in
10 admixture with a pharmaceutically acceptable adjuvant, for
11 example normal saline, a lipid emulsion, albumen,
12 phosphate buffered saline or the like and are administered
13 in an amount effective to mediate treatment of said
14 cancerous disease, for example with a range of about 1
15 microgram per mil to about 1 gram per mil. In a
16 particular embodiment, the anti-cancer antibodies useful
17 in either of the above outlined methods may be a humanized
18 antibody.

CLAIMS

What is claimed is:

Claim 1. A method for the production of patient specific anti-cancer antibodies which are useful in treating a cancerous disease comprising:

obtaining a tissue sample from a particular individual;

manufacturing antibodies directed against cells of said tissue sample;

subjecting said manufactured antibodies to a cytotoxicity assay designed to produce a subset of antibodies which express an enhanced degree of cytotoxicity directed toward cancer cells;

whereby said subset of antibodies defines a group of patient specific anti-cancer antibodies characterized as being cytotoxic to cancer cells and relatively benign toward normal cells.

Claim 2. The patient specific anti-cancer antibodies or fragments thereof produced in accordance with the method of claim 1.

Claim 3. The patient specific anti-cancer antibodies or fragments thereof in accordance with claim 2 wherein the cytotoxicity of said antibodies is mediated through antibody dependent cellular toxicity.

Claim 4. The patient specific anti-cancer antibodies or fragments thereof in accordance with claim 2 wherein the cytotoxicity of said antibodies is mediated through complement dependent cellular toxicity.

Claim 5. The patient specific anti-cancer antibodies or fragments thereof in accordance with claim 2 wherein the cytotoxicity of said antibodies is mediated through catalyzing of the hydrolysis of cellular chemical bonds.

1 Claim 6. The patient specific anti-cancer antibodies
2 or fragments thereof in accordance with claim 2 wherein
3 the cytotoxicity of said antibodies is mediated through
4 producing an immune response against putative cancer
5 antigens residing on tumor cells.

6
7 Claim 7. The patient specific anti-cancer antibodies
8 or fragments thereof in accordance with claim 2 wherein
9 the cytotoxicity of said antibodies is mediated through
10 targeting of cell membrane proteins to interfere with
11 their function.

12
13 Claim 8. The patient specific anti-cancer antibodies
14 or fragments thereof in accordance with claim 2 wherein
15 the cytotoxicity of said antibodies is mediated through
16 production of a conformational change in a cellular
17 protein effective to produce a signal to initiate cell-
18 killing.

19
20 Claim 9. A method for treating a patient suffering
21 from a cancerous disease comprising:

22 administering to said patient the patient specific
23 anti-cancer antibodies of claim 2,

24 wherein said anti-cancer antibodies are in admixture
25 with a pharmaceutically acceptable adjuvant and are
26 administered in an amount effective to mediate treatment
27 of said cancerous disease.

28
29 Claim 10. The method of claim 9, wherein the patient
30 specific anti-cancer antibody is a humanized antibody.

31
32 Claim 11. A method for treating a patient suffering
33 from a cancerous disease comprising:

34 conjugating said patient specific anti-cancer
35 antibodies of claim 2 with a member selected from the
36 group consisting of toxins, enzymes, radioactive
37 compounds, and hematogenous cells; and

1 administering conjugated antibodies to said patient;
2 wherein said anti-cancer antibodies are in admixture
3 with a pharmaceutically acceptable adjuvant and are
4 administered in an amount effective to mediate treatment
5 of said cancerous disease.

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7 Claim 12. The method of claim 11, wherein the patient
8 specific anti-cancer antibody is a humanized antibody.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K16/30 A61K39/395 A61P35/00 A61K47/48 A61K51/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 222 360 A (BIOTHERAPEUTICS, INC.) 20 May 1987 (1987-05-20) the whole document ---	1-12
Y	US 5 869 045 A (HELLSTROM ET AL.) 9 February 1999 (1999-02-09) cited in the application examples ---	1-12
A	US 4 939 240 A (CHU ET AL.) 3 July 1990 (1990-07-03) paragraph 6.6.4 paragraph 6.11.3 tables II, XIII ---	1-12
A	WO 94 18345 A (AFFYMAX TECHNOLOGIES NV) 18 August 1994 (1994-08-18) page 51, line 16 -page 57, line 26 -----	1-12



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

19 June 2001

Date of mailing of the international search report

18/07/2001

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Nooij, F

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/IB 00/02050**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 9-12 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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